

What is Claimed Is:

1. A method for detecting the production of a primer extension product in a primer extension reaction mixture, said method comprising:

(a) producing a primer extension mixture that includes a nucleic acid polymerase having 3'→5' exonuclease activity and a FET labeled oligonucleotide that includes a 3'→5' exonuclease resistant quencher domain;

(b) subjecting said primer extension mixture to primer extension reaction conditions;

(c) detecting a change in a fluorescent signal from said FET labeled oligonucleotide probe to obtain an assay result; and

(d) employing said assay result to determine whether a primer extension product is present in said mixture.

2. The method according to Claim 1, wherein said primer extension reaction is a PCR amplification reaction.

3. The method according to Claim 2, wherein said method is a real-time method of monitoring said PCR amplification reaction.

4. The method according to Claim 1, wherein said FET labeled oligonucleotide is a nucleic acid detector molecule that includes a single- stranded target binding sequence linked to fluorophore and dark quencher.

5. The method according to Claim 4, wherein energy transfer occurs between said fluorophore and dark quencher of said FET labeled oligonucleotide probe upon fluorophore excitation when said FET labeled oligonucleotide is not hybridized to target nucleic acid.

6. The method according to Claim 5, wherein energy transfer does not occur between said fluorophore and dark quencher of said FET labeled oligonucleotide probe upon fluorophore excitation when said FET labeled oligonucleotide probe is hybridized to a target nucleic acid.

7. The method according to Claim 3, wherein said method is a 5' nuclease method of monitoring a PCR amplification reaction.

8. The method according to Claim 7, wherein energy transfer does not occur between said fluorophore and dark quencher of said FET labeled oligonucleotide probe upon fluorophore excitation when said FET labeled oligonucleotide probe is cleaved by 5' nuclease.

9. The method according to Claim 4, wherein said target binding sequence comprises a hybridization domain complementary to a sequence of said primer extension product.

10. A method of monitoring of a PCR amplification reaction, said method comprising:

- (a) preparing a PCR amplification reaction mixture by combining:
 - (i) a template nucleic acid;
 - (ii) forward and reverse nucleic acid primers;
 - (iii) deoxyribonucleotides;
 - (iv) a nucleic acid polymerase having 3'→5' exonuclease activity; and
 - (v) a FET labeled oligonucleotide that includes: a 3'→5' exonuclease

resistant quencher domain comprising a dark quencher, a fluorescent reporter domain comprising a fluorophore and a PCR product complementary domain, wherein fluorescence energy transfer does not occur between said fluorophore and said quencher upon fluorophore excitation when said FET labeled oligonucleotide is hybridized to a product nucleic acid of said PCR reaction;

(b) subjecting said PCR amplification reaction mixture to PCR amplification conditions;

(c) monitoring said reaction mixture for a fluorescent signal from said FET labeled oligonucleotide probe to obtain an assay result; and

(d) employing said assay result to monitor said PCR amplification reaction.

11. The method according to Claim 10, wherein said method is a method of monitoring a PCR amplification reaction in real time.

12. The method according to Claim 10, wherein said FET labeled oligonucleotide is a probe selected from the group consisting of: scorpion probes, sunrise probes, molecular beacons, and conformationally assisted probes.

13. The method according to Claim 10, wherein said fluorescence energy transfer occurs between said fluorophore and quencher of said FET labeled oligonucleotide upon fluorophore excitation when said FET labeled oligonucleotide is not hybridized to said product nucleic acid.

14. The method according to Claim 10, wherein said dark quencher is located at the 3' end of said FET labeled oligonucleotide.

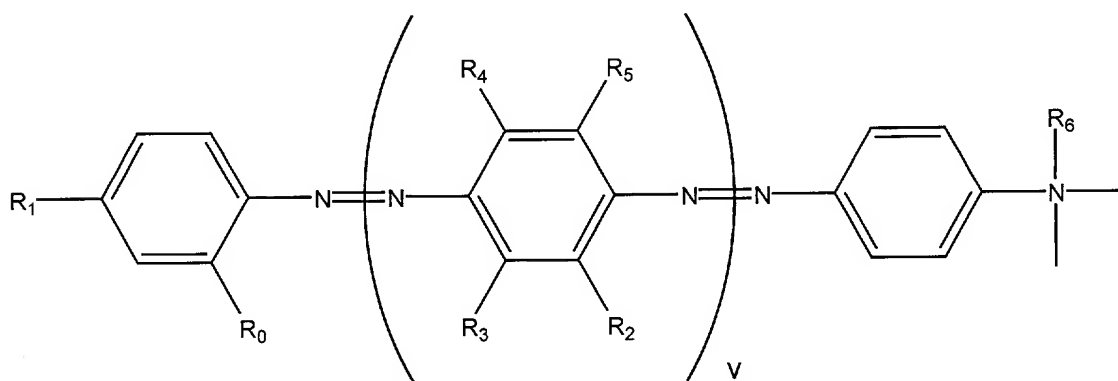
15. The method according to Claim 14, wherein said dark quencher has maximum absorbance between about 400 and about 700 nm.

16. The method according to Claim 15, wherein said dark quencher has maximum absorbance between about 500 and about 600 nm.

17. The method according to Claim 16, wherein said dark quencher comprises a substituted 4-(phenyldiazenyl)phenylamine structure.

18. The method according to Claim 16, wherein said dark quencher has a structure comprising at least two residues selected from aryl, substituted aryl, heteroaryl, substituted heteroaryl and combination thereof, wherein at least two of said residues are covalently linked via an exocyclic diazo bond.

19. The method according to Claim 18, wherein said dark quencher comprises a formula:



wherein:

$R_0, R_1, R_2, R_3, R_4, R_5$ are independently: $-H$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, SO_3 , $-N[(CH_2)_nCH_3]_2$ wherein $n=0$ to 5 or $-CN$;
 R_6 is $-H$ or $-(CH_2)_nCH_3$ where $n=0$ to 5 ; and
 v is a number from 0 to 10 .

20. A method of monitoring of a PCR amplification reaction, said method comprising:

- (a) preparing a PCR amplification reaction mixture by combining:
 - (i) a template nucleic acid;
 - (ii) forward and reverse nucleic acid primers;
 - (iii) deoxyribonucleotides;

- (iv) a nucleic acid polymerase having 3'→5' exonuclease and 5'→3' exonuclease activity; and
 - (v) a FET labeled oligonucleotide that includes: a 3'→5' exonuclease resistant quencher domain comprising a dark quencher, a fluorescent reporter domain comprising a fluorophore and a PCR product complementary domain, where fluorescence energy transfer occurs between said fluorophore and quencher upon fluorophore excitation when said FET labeled oligonucleotide is not hybridized to said template nucleic acid;
 - (b) subjecting said PCR amplification reaction mixture to PCR amplification conditions;
 - (c) monitoring said reaction mixture for a fluorescent signal from said FET labeled oligonucleotide probe to obtain an assay result; and
 - (d) employing said assay result to monitor said PCR amplification reaction.
21. The method according to Claim 20, wherein said method is a method of monitoring a PCR amplification reaction in real-time.
22. The method according to Claim 20, wherein said fluorescence energy transfer does not occur between said fluorophore and quencher of said FET labeled oligonucleotide upon fluorophore excitation when said FET labeled oligonucleotide is hybridized to said product nucleic acid.
23. The method according to Claim 20, wherein said fluorescence energy transfer does not occur between said fluorophore and quencher of said FET labeled oligonucleotide upon fluorophore excitation when said FET labeled oligonucleotide is cleaved by 5' nuclease.
24. The method according to Claim 20, wherein said quencher is located at the 3' end of said FET oligonucleotide.

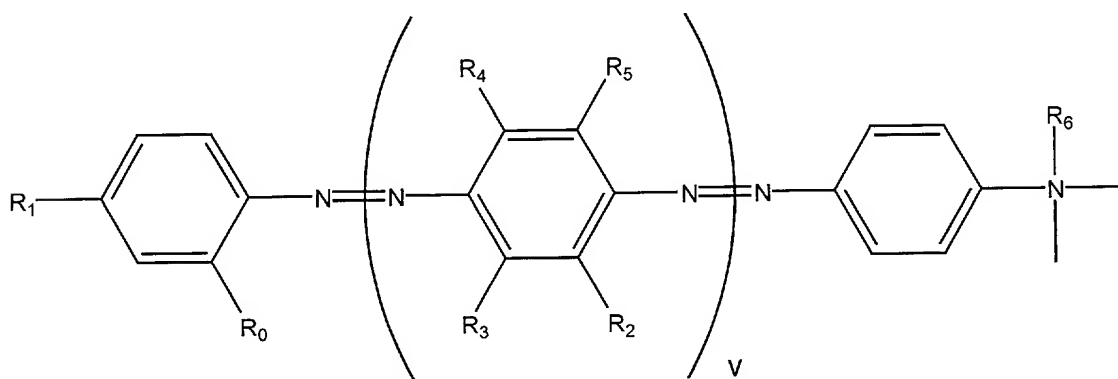
25. The method according to Claim 24, wherein said dark quencher has maximum absorbance between about 400 and about 700 nm.

26. The method according to Claim 25, wherein said dark quencher has a maximum absorbance between about 500 and about 600 nm.

27. The method according to Claim 26, wherein said dark quencher comprises a substituted 4-(phenyldiazenyl)phenylamine structure.

28. The method according to Claim 26, wherein said dark quencher has a structure comprising at least two residues selected from aryl, substituted aryl, heteroaryl, substituted heteroaryl and combination thereof, wherein at least two of said residues are covalently linked via an exocyclic diazo bond.

29. The method according to Claim 28, wherein said dark quencher has the following structure:



wherein:

R₀, R₁, R₂, R₃, R₄, R₅ are independently: -H, halogen, -O(CH₂)_nCH₃, -(CH₂)_nCH₃, -NO₂, SO₃, -N[(CH₂)_nCH₃]₂ wherein n=0 to 5 or -CN;

R₆ is -H or -(CH₂)_nCH₃ where n=0 to 5; and

v is a number from 0 to 10.

30. A method for screening a nucleic acid sample for the presence of first and second nucleic acids that differ from each other by a single nucleotide, said method comprising:

- (a) producing a primer extension mixture that includes:
 - (i) said nucleic acid sample;
 - (ii) a nucleic acid polymerase having 3'→5' exonuclease activity; and
 - (iii) first and second FET labeled oligonucleotide probes that are complementary to said first and second nucleic acids, respectively, wherein each of said first and second FET labeled oligonucleotides includes a 3'→5' exonuclease resistant quencher domain;

(b) subjecting said primer extension mixture to primer extension reaction conditions;

(c) detecting a change in a fluorescent signal, if any, from said first and second FET labeled oligonucleotide probes to obtain an assay result; and

(d) employing said assay result to determine the presence or absence of said first and second nucleic acids in said sample.

31. The method according to Claim 30, wherein said FET labeled oligonucleotide probes are nucleic acid detector molecules that include a single- stranded target binding sequence linked to fluorophore and dark quencher.

32. The method according to Claim 31, wherein energy transfer occurs between said fluorophore and dark quencher of each of said FET labeled oligonucleotide probes upon fluorophore excitation when said FET labeled oligonucleotide is not hybridized to target nucleic acid.

33. The method according to Claim 31, wherein energy transfer does not occur between said fluorophore and dark quencher of said FET labeled oligonucleotide probe upon fluorophore excitation when said FET labeled oligonucleotide probe is hybridized to a target nucleic acid.

34. The method according to Claim 31, wherein energy transfer does not occur between said fluorophore and dark quencher of said FET labeled oligonucleotide probes upon fluorophore excitation when said FET labeled oligonucleotide probe is cleaved by 5' nuclease.

35. The method according to Claim 30, wherein said second nucleic acid is an SNP of said first nucleic acid.

36. A system for use in detecting the production of a primer extension product in a primer extension reaction mixture, said system comprising:

- (a) a FET labeled oligonucleotide that includes a 3'→5' exonuclease resistant quencher domain; and
- (b) a nucleic acid polymerase having 3'→5' exonuclease activity.

37. The system according to Claim 36, wherein said FET labeled oligonucleotide has said quencher domain located at its 3' end.

38. The system according to Claim 37, wherein said quencher domain comprises a dark quencher.

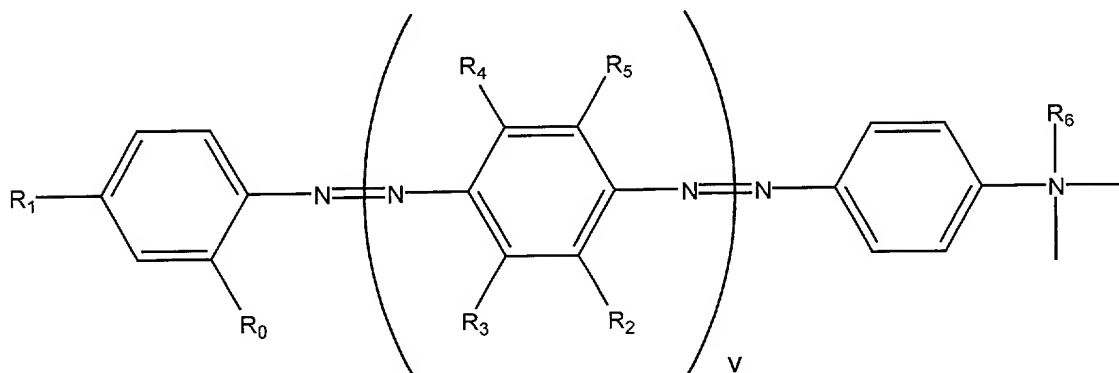
39. The system according to Claim 38, wherein said dark quencher has maximum absorbance between about 400 and about 700 nm.

40. The system according to Claim 39, wherein said dark quencher has a maximum absorbance between about 500 and 600 nm.

41. The system according to Claim 40, wherein said dark quencher comprises a substituted 4-(phenyldiazenyl)phenylamine structure.

42. The system according to Claim 40, wherein said dark quencher has a structure comprising at least two residues selected from aryl, substituted aryl, heteroaryl, substituted heteroaryl and combination thereof, wherein at least two of said residues are covalently linked via an exocyclic diazo bond.

43. The system according to Claim 42, wherein said dark quencher has the following structure:



wherein:

R₀, R₁, R₂, R₃, R₄, R₅ are independently: -H, halogen, -O(CH₂)_nCH₃, -(CH₂)_nCH₃, -NO₂, SO₃, -N[(CH₂)_nCH₃]₂ wherein n=0 to 5 or -CN;
R₆ is -H or -(CH₂)_nCH₃ where n=0 to 5; and
v is a number from 0 to 10.

44. A kit for use in detecting the production of a primer extension product in a primer extension reaction mixture, said kit comprising:

(a) a FET labeled oligonucleotide that includes a 3'→5' exonuclease resistant quencher domain; and

(b) instructions for practicing the method according to Claim 1.

45. The kit according to Claim 44, wherein said kit further comprises a nucleic acid polymerase having 3'→5' exonuclease activity.

46. The kit according to Claim 44, wherein said FET labeled oligonucleotide has said quencher domain located at its 3' end.

47. The kit according to Claim 46, wherein said quencher domain comprises a dark quencher.

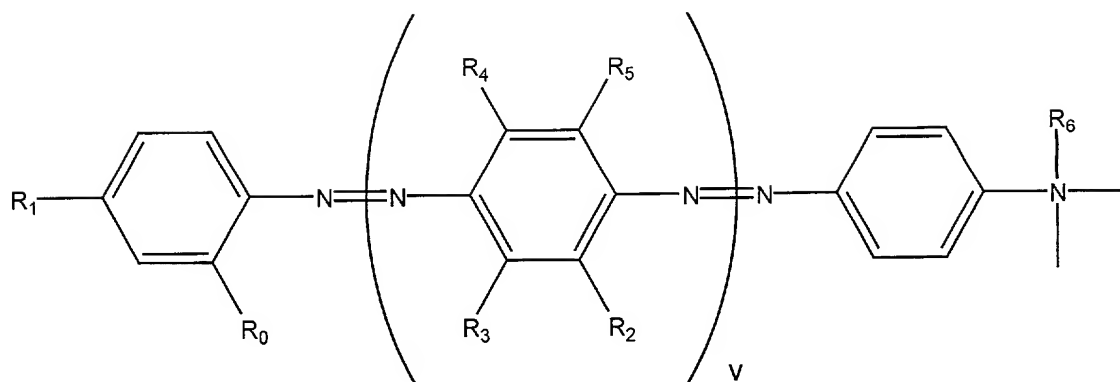
48. The kit according to Claim 47, wherein said dark quencher has maximum absorbance between about 400 and about 700 nm.

49. The kit according to Claim 48, wherein said dark quencher has a maximum absorbance between about 500 and 600 nm.

50. The kit according to Claim 49, wherein said dark quencher comprises a substituted 4-(phenyldiazenyl)phenylamine structure.

51. The kit according to Claim 49, wherein said dark quencher has a structure comprising at least two residues selected from aryl, substituted aryl, heteroaryl, substituted heteroaryl and combination thereof, wherein at least two of said residues are covalently linked via an exocyclic diazo bond.

52. The kit according to Claim 51, wherein said dark quencher has the following structure:



wherein:

$R_0, R_1, R_2, R_3, R_4, R_5$ are independently: $-H$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, SO_3 , $-N[(CH_2)_nCH_3]_2$ wherein $n=0$ to 5 or $-CN$;
 R_6 is $-H$ or $-(CH_2)_nCH_3$ where $n=0$ to 5 ; and
 v is a number from 0 to 10 .

53. In a method comprising employing fluorescently labeled nucleic acid detector and a polymerase having a $3' \rightarrow 5'$ exonuclease activity, the improvement comprising:

employing a FET labeled oligonucleotide that includes a $3' \rightarrow 5'$ exonuclease resistant quencher domain as said fluorescently labeled nucleic acid detector.

or

employing a FET labeled oligonucleotide that includes a $3' \rightarrow 5'$ exonuclease resistant donor domain as said fluorescently labeled nucleic acid detector.

54. The method according to Claim 53, wherein said FET labeled oligonucleotide has said quencher domain located at its $3'$ end.

55. The method according to Claim 54, wherein said quencher domain comprises a dark quencher.

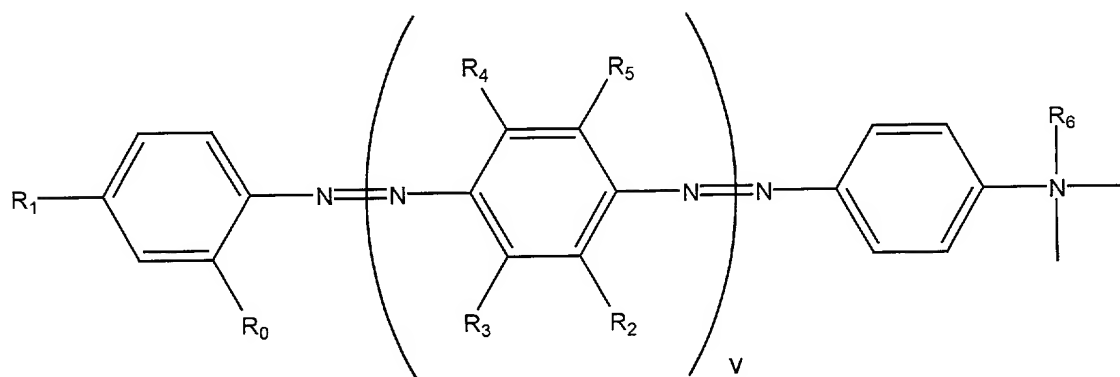
56. The method according to Claim 55, wherein said dark quencher has maximum absorbance between about 400 and about 700 nm.

57. The method according to Claim 56, wherein said dark quencher has a maximum absorbance between about 500 and 600 nm.

58. The method according to Claim 57, wherein said dark quencher comprises a substituted 4-(phenyldiazenyl)phenylamine structure.

59. The method according to Claim 57, wherein said dark quencher has a structure comprising at least two residues selected from aryl, substituted aryl, heteroaryl, substituted heteroaryl and combination thereof, wherein at least two of said residues are covalently linked via an exocyclic diazo bond.

60. The method according to Claim 59, wherein said dark quencher has the following structure:



wherein:

$R_0, R_1, R_2, R_3, R_4, R_5$ are independently: $-H$, halogen, $-O(CH_2)_nCH_3$, $-(CH_2)_nCH_3$, $-NO_2$, SO_3 , $-N[(CH_2)_nCH_3]_2$ wherein $n=0$ to 5 or $-CN$;

R_6 is $-H$ or $-(CH_2)_nCH_3$ where $n=0$ to 5; and

v is a number from 0 to 10.

20200624